Document made available under the Patent Cooperation Treaty (PCT)

International application number: PCT/US05/009228

International filing date: 21 March 2005 (21.03.2005)

Document type: Certified copy of priority document

Document details: Country/Office: US
Number: 60/554,790
Filing date: 19 March 2004 (19.03.2004)

Date of receipt at the International Bureau: 25 April 2005 (25.04.2005)

Remark: Priority document submitted or transmitted to the International Bureau in compliance with Rule 17.1(a) or (b)

World Intellectual Property Organization (WIPO) - Geneva, Switzerland
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APPLICATION NUMBER: *60/554,790*
FILING DATE: *March 19, 2004*
RELATED PCT APPLICATION NUMBER: *PCT/US05/09228*

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TITLE OF THE INVENTION (280 characters max)

LIPOSOMAL CARBOHYDRATE DERIVATIVES FOR TARGETING OF CELLS EXPRESSING C-TYPE LECTIN SURFACE RECEPTORS AND INTRACELLULAR DELIVERY OF DEFINED LECTINS INHIBITING INTRACELLULAR PATHOGENS VIA INTERACTION WITH PATHOGEN SURFACE CARBOHYDRATE MOIETIES

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ENCLOSED APPLICATION PARTS *(check all that apply)*

☒ Specification *Number of Pages* 16 ☐ CD(s), Number [ ]

☒ Drawing(s) *Number of Sheets* 2 ☐ Other (specify) [ ]

☐ Application Data Sheet. See 37 CFR 1.76

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Respectfully submitted,

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Date 3/19/04

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U.S. PROVISIONAL PATENT APPLICATION
OF
ROBERT K. GIESELER
AND
MICHAEL J. SCOLARO

FOR

UNITED STATES LETTERS PATENT

ON

LIPOSOMAL CARBOHYDRATE DERIVATIVES FOR TARGETING OF CELLS EXPRESSING C-TYPE LECTIN SURFACE RECEPTORS AND INTRACELLULAR DELIVERY OF DEFINED LECTINS INHIBITING INTRACELLULAR PATHOGENS VIA INTERACTION WITH PATHOGEN SURFACE CARBOHYDRATE MOIETIES

Docket: 23046-81250
Sheets of Drawings: 2

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LIPOSOMAL CARBOHYDRATE DERIVATIVES FOR TARGETING OF CELLS EXPRESSING C-TYPE LECTIN SURFACE RECEPTORS AND INTRACELLULAR DELIVERY OF DEFINED LECTINS INHIBITING INTRACELLULAR PATHOGENS VIA INTERACTION WITH PATHOGEN SURFACE CARBOHYDRATE MOIETIES

BACKGROUND OF THE INVENTION

1. Field of the Invention

The present invention relates to the medical arts, and in particular, to targeted liposomal delivery of active agents.

2. Discussion of the Related Art

Professional antigen-presenting cells (APCs) comprise three major classes of immune cells, namely dendritic cells (DCs), follicular dendritic cells (FDCs) and macrophages (MPs). In the the mammalian organism, these cells are located both in non- lymphoid organs and tissues (certain subsets of immature DCs and certain subsets of MPs), as well as lymphoid organs and tissues (certain subsets of mature DCs [which are also termed interdigitating DCs, or IDCs], certain subsets of MPs, and FDCs) (reviewed in Imai Y, Yamakawa M, Kasajima T. The lymphocyte-dendritic cell system. Histol Histopathol. 1998 Apr;13(2):469-510).

Among the professional APCs, DCs are the most potent stimulators of antigen-specific immunity, and they also have the unique ability to induce primary cellular and humoral immune responses (such as, for example, in the intestinal mucosa, as reviewed in Telemo E, Korotkova M, Hanson LA. Antigen presentation and processing in the intestinal mucosa and lymphocyte homing. Ann Allergy Asthma Immunol 2003 Jun;90(6 Suppl 3):28-33, or in the microenvironment of the eye, i.e. Novak N, Siepmann K, Zierhut M, Bieber T. The good, the bad and the ugly – APCs of the eye. Trends Immunol 2003 Nov;24(11):570-4). In MPs, antigen presentation is one of several functions these cells display. Collectively, MPs are responsible for secondary antigen-specific responses that had earlier been initiated by DCs (such as, for example, in the brain, as reviewed in Thomas WE. Brain macrophages: on the role of pericytes and perivascular cells. Brain Res Brain Res Rev. 1999 Dec;31(1):42-57). Finally, the presence of FDCs is – at least under healthy

conditions – tightly restricted to the lymphoid follicles within lymphoid organs and tissues where they are instructing B lymphocytes for the production of antigen-specific immunoglobulins. Importantly, these cells also provide potent long-term memory for antigens or whole viruses by retaining these structures within their abundant cytoplasmic projections (reviewed in van Nierop K, de Groot C. Human follicular dendritic cells: function, origin and development. Semin Immunol. 2002 Aug;14(4):251-7). Of note, both DCs and MPs are the principal inducers of T-cell (or cellular) immunity, while FDCs are the sole inducers of B-cell (or humoral) immunity.

In context of the invention described herein it is to be stressed that professional APCs provide a long-term sanctuary for viruses such as the human immunodeficiency virus 1 (HIV-1) and the hepatitis C virus (HCV). As discussed for the example of HIV-1, this virus is preserved in both cellular and anatomical sanctuaries. Cellular sanctuaries include DCs containing intracytoplasmically stored highly infectious virus as well as membrane-bound HIV-1; MPs expressing HIV-1 for prolonged periods; FDCs retaining infectious HIV-1 packaged within their outer membranes for indeterminate lengths of time; and memory T cells with integrated HIV-1 proviral DNA. The key anatomical reservoir for HIV-1 appears to be the central nervous system (reviewed in Schrager LK, D'Souza MP. Cellular and anatomical reservoirs of HIV-1 in patients receiving potent antiretroviral combination therapy. JAMA. 1998 Jul 1;280(1):67-71; Burton GF, Keele BF, Estes JD, Thacker TC, Gartner S. Follicular dendritic cell contributions to HIV pathogenesis. Semin Immunol. 2002 Aug;14(4):275-84; and Gieseler RK, Marquitan G, Scolaro MJ, Cohen MD. Lessons from history: dysfunctional APCs, inherent dangers of STI and an important goal, as yet unmet. Trends Immunol. 2003 Jan;24(1):11). In order to conceive and devise causative therapies for HIV disease and other viral diseases where such sanctuaries are established, it must be the ultimate goal to eliminate the respective virus from its cellular sanctuaries in the infected persons.

Many, if not all, features ultimately leading to the establishment of cellular reservoirs for pathogens relate to evolutionarily old biological mechanisms of defense. In 1988, Kurt Drickamer summarized the advances in the field of animal lectins (i.e., sugar-binding proteins) that carry defined carbohydrate recognition domains (CRDs) (reviewed in Drickamer K. Two distinct classes

of carbohydrate-recognition domains in animal lectins. J Biol Chem 1988 Jul 15;263(20):9557-60). Today we know that these lectins are part of our ancient immunological heritage and can, for example, be found in species as distant as flies and humans (Hallman M, Ramet M, Ezekowitz RA. Toll-like receptors as sensors of pathogens. Pediatr Res 2001 Sep;50(3):315-21). In evolutionary terms, the archaic and more robust branch termed *innate immunity* evolved long before the development of the more sophisticated *adaptive immunity* that is based on the expansion of cellular clones providing us with the luxury of antigen-specific defense responses. Nevertheless, innate immunity – including animal lectins and their ligands – still is the foundation of every developmentally advanced immune system and even plays an important role in the clonal immune response (Holmskov U, Thiel S, Jensenius JC. Collections and ficolins: humoral lectins of the innate immune defense. Annu Rev Immunol 2003;21:547-78. Epub 2001 Dec 19). As is discussed below in detail, these ancient lectins play a central role in the establishment of pathogen reservoirs – and thus, in the current incurablity of some of the most prevalent and lethal human diseases. This current invention thus materializes in an immunological strategy that is based on the primordial fundamental lectin concept.

While biochemists had already accumulated profound knowledge on CRD receptors, immunology first realized, on a theoretical construct, archaic "broad-spectrum receptors" as soon as Charles A. Janeway introduced his Infectious-Nonself Model, proposing that APCs initiate protective immunity when triggered by pathogen-associated molecular patterns (PAMPs) *via* pattern recognition receptors (PRRs) (Janeway Jr, CA Approaching the asymptote? Evolution and revolution in immunology. Cold Spring Harbor Symp Quant Biol 1989;54:1–13). These two theoretically predicted structures were successively discovered experimentally. Owing to their evolutionary anchestor, the PRRs were then termed Toll-like receptors (TLRs) (reviewed in Johnson, GB *et al.* Evolutionary clues to the functions of the Toll-like family as surveillance receptors. Trends Immunol 2003 24, 19–24).

A subgroup of the CRD lectins is the so-called C-type lectin receptors (CTLRs) characterized by displaying a C-type lectin domain (CTLD) that binds carbohydrates in calcium-dependent manner (Holmskov U, Malhotra R, Sim RB, Jensenius JC. Collectins: collagenous C-

type lectins of the innate immune defense system. Immunol Today. 1994 Feb;15(2):67-74). One example for CTLD receptors recognizing PAMPs are the so-called collectins that are present in plasma and on mucosal surfaces. Human collectins thus far identified are the mannan-binding lectin (MBL) and the surfactant proteins A and D (SP-A and SP-D). When recognizing an infectious agent, they either (MBL) initiate the lectin pathway of complement activation or (SP-A, SP-D) trigger opsonization, neutralization, and agglutination to limit infection and orchestrate the adaptive immune response (reviewed in Holmskov U, Thiel S, Jensenius JC. Collections and ficolins: humoral lectins of the innate immune defense. Annu Rev Immunol. 2003;21:547-78. Epub 2001 Dec 19). A second example for CTLD receptors is the Regenerating Gene (Reg or REG) Family that mainly plays a protective role in the hepatogastroenterological organs and tissues (with currently 17 cloned and sequenced family members). These regional CTLD receptors are involved in injury, inflammation, diabetes and carcinogenesis. The role of some of these receptors in carcinogenesis makes them promising candidate molecules as new prognostic indicators of tumor survival, early biomarkers of carcinogenesis, and molecular matrices for the design of novel chemotherapeutics (reviewed in Zhang YW, Ding LS, Lai MD. Reg gene family and human diseases. World J Gastroenterol. 2003 Dec;9(12):2635-41).

For example, pathogens are recognized by TLRs and C-type lectins expressed on the surface of DCs. However, some pathogens, including HIV-1 and *Mycobacterium tuberculosis*, subvert DC functions to escape immune surveillance by targeting the DC-specific intercellular adhesion molecule-grabbing nonintegrin (DC-SIGN, CD209), a C-type lectin. Such pathogens misuse DC-SIGN by distinct mechanisms that either circumvent antigen processing or alter TLR-mediated signalling whereby skewing T-cell responses (reviewed in van Kooyk Y, Geijtenbeek TB. DC-SIGN: escape mechanism for pathogens. Nat Rev Immunol. 2003 Sep;3(9):697-709). In addition, other C-type lectin receptors such as Langerin (which is exclusively expressed by the epidermal and mucosal DC subset termed Langerhans cells) and on the dermal DC subset's mannose receptor (MR, CD206) are at least equally important for HIV-1 gp120 binding on epithelial DCs. Importantly, C-type lectin receptors play a role in virus transfer to T cells, either via de novo infection (*cis* transfer) or without infection (*in-trans* or *trans* infection) (reviewed in Turville S, Wilkinson J, Cameron P, Dable J, Cunningham AL. The role of dendritic cell C-type lectin

receptors in HIV pathogenesis. J Leukoc Biol. 2003 Nov;74(5):710-8. Epub 2003 Sep 02), and they play a role in the uptake and intracellular storage of highly infectious HIV-1 (Gieseler RK, Marquitan G, Hahn MJ, Perdon LA, Driessen WHP, Sullivan SM, Scolaro MJ. DC-SIGN-specific liposomal targeting and selective intracellular compound delivery to human myeloid dendritic cells: implications for HIV disease. Scand J Immunol 2004: in press; and references therein). It has been shown that these types of CTLD receptors bind sugars that are predominantly expressed by pathogens. Therefore, they show preferential and selective binding of the monosaccharides mannose, fucose, and N-acetylglucosamine, but display much higher affinity for multivalent oligosaccharides of similar nature, such as those found on the surface of potentially pathogenic microorganisms (reviewed in Taylor ME, Drickamer K. Structural requirements for high affinity binding of complex ligands by the macrophage mannose receptor. J Biol Chem. 1993 Jan 5;268(1):399-404). These receptors may thus be harnessed for shuttling liposomes labeled with certain mono- or polysaccharides (such as fucose or polymerized fucose) into intracellular compartments exploited by pathogens (viruses, bacteria, or fungi). In addition, liposomes thus shuttled into intracellular compartments (as, for example, endosomes) may deliver lectins that, will be suitable to agglutinate intracellularly stored pathogens (such as HIV-1 or HCV), so as to generate large lectin-pathogen complexes that may be easily recognized by the infected cell and, subsequently, be degraded enzymatically and/or pH-dependently. For example, one lectin that appears highly suitable for this purpose when addressing the HIV-1 reservors is the *Myrianthus holstii* lectin (MHL, a.k.a. Myrianthin) which is obtained from the roots of the Tanzanian plant *Myrianthus holstii*. MHL comprises several most favorable characteristics, namely agglutination of HIV-1; no toxicity for greater than two orders of magnitude above the effective dosage in 50% of infected cells (EC_{50}); and the lack of mitogenicity for human leukocytes (Charan RD, Munro MH, O'Keefe BR, Sowder RCII, McKee TC, Currens MJ, Pannell LK, Boyd MR. Isolation and characterization of *Myrianthus holstii* lectin, a potent HIV-1 inhibitory protein from the plant *Myrianthus holstii*. J Nat Prod 2000 Aug;63(8):1170-4).

SUMMARY OF THE INVENTION

The present invention relates to liposomes and methods of preferentially, or "actively," targeting and delivering an active agent, such as a lectin or drug, to a mammalian immune cell in vivo or in vitro. In particular, the present invention describes liposomes targeted by surface-derivatized mono- or poly-fucose (or, alternatively, chemically related or chemically modified sugars) that comprise, incorporate or encapsulate a lectin, or lectins, such as, but not limited to, lectins of the African plant *Myrianthus holstii* (i.a. MBL), to a host of pathogen reservoir cells in HIV disease, HCV-related hepatitis, *Mycobacterium tuberculosis*, and various other disease entities coinciding with the formation of intracellular pathogen reservoirs. These cellular sanctuaries include the different immunological, developmental, and anatomical subsets of dendritic cells, macrophages, and follicular dendritic cells. Liposomal cell-specific targeting is to be achieved *via* evolutionarily conserved receptors expressing carbohydrate recognition domains (CRDs), and specifically, the so-called C-type lectin receptors (CTLRs), including MR (CD206), langerin (CD207), DEC-205 (CD205), and DC-SIGN (CD209), all of which are so-called multilectin receptors. The objective of this invention is to provide a means for eradicating intracellular pathogen reservoirs, thus providing a most valuable novel approach for the therapy of a great variety of viral, bacterial, and fungal disease entities. Some embodiments of the invention can also be applied for the treatment of malignant diseases, especially those relating to the liver and the gastrointestinal tract.

A benefit of the invention is that it permits targeting of all major known HIV reservoir cell types. This comes at an extremely low cost, and the invention can be applied to the treatment of a host of other diseases.

DETAILED DESCRIPTION OF THE PREFERRED EMBODIMENTS

Some embodiments of the present invention are directed to a method of preferentially delivering an active agent to an immune cell of a mammalian subject, including a human. The targeted immune cells include myeloid progenitor cells, dendritic cells, monocytes, macrophages, or T-lymphocytes. A dendritic cell includes a myeloid dendritic cell, a plasmacytoid dendritic cell, or

a follicular dendritic cell. A T-lymphocyte includes, but is not limited to, a T-helper cell or a T-memory cell.

The term "preferentially" means that the lipid-active agent complex, or the liposome, is delivered to the cell and the active agent (e.g., the plant lectin or drug) is taken up by the cell, more effectively than delivery and uptake of the agent using a comparable lipid-active agent complex, or liposome, having an outer surface that does not comprise mono- or poly-fucose moieties, in contrast with the invention.

The method involves injecting into the mammalian subject a targeted liposome in accordance with the invention, that comprises the active agent or combination of active agents, the immune cell being infected with, or susceptible to infection with, an infectious agent, such as but not limited to human immunodeficiency virus (HIV). In some embodiments, the immune cell is infected with, or susceptible to infection with, an infectious agent, such as a virus, a bacterium, a fungus, a protozoan, or a prion. Examples of viral infectious agents are HIV, HSV, EBV, and HPV. Some embodiments are particularly directed to intracellular targeting for intraendosomal HIV inactivation.

A "complex" is a mixture or adduct resulting from chemical binding or bonding between and/or among its constituents or components, including the lipid, active agent, and other optional components of the inventive lipid-active agent complex. Chemical binding or bonding can have the nature of a covalent bond, ionic bond, hydrogen bond, Van der Waal's bond, hydrophobic bond, or any combination of these bonding types linking the constituents of the complex at any of their parts or moieties, of which a constituent can have one or a multiplicity of moieties of various sorts. Not every constituent of a complex need be bound to every other constituent, but each constituent has at least one chemical bond with at least one other constituent of the complex. In accordance with the present invention, examples of lipid-active agent complexes include liposomes (lipid vesicles), or lipid-active agent sheet-disk complexes. Lipid-conjugated active agents can also be a part of the lipid-active agent complex in accordance with the invention.

The present invention is also directed to inventive targeted liposomes including at their outer surfaces mono- or poly-fucose, attached to the liposomes by known techniques such as has been

presented earlier, that is by the preparation of fucosylated (Fuc) liposomes by means of cholesterol linkeage and membrane anchoring with cholesten-5-yloxy-N-(4-((1-imino-2-beta-D-thiofucosyle thyl)amino)alkyl)formamide (or, briefly, Fuc-C4-Chol), and the subsequent formulation of liposomes as a composition of distearoylphosphatidylcholine (DSPC), cholesterol (Chol), and Fuc-C4-Chol at a molar ratio of 60:35:5 (Kawakami S, Wong J, Sato A, Hattori Y, Yamashita F, Hashida M. Biodistribution characteristics of mannosylated, fucosylated, and galactosylated liposomes in mice. Biochim Biophys Acta. 2000 Dec 15;1524(2-3):258-65).

Useful techniques for making lipid-active agent complexes, such as liposomes, are known in the art. (E.g., Sullivan SM, Gieseler RKH, Lenzner S, Ruppert J, Gabrysiak TG, Peters JH, Cox G, Richer, L, Martin, WJ, and Scolaro, MJ, Inhibition of Human Immunodeficiency Virus-1 Proliferation by Liposome-Encapsulated Sense DNA to the 5' TAT Splice Acceptor Site, Antisense Research and Development 2:187-197 [1992]; Laverman P, Boerman OC, Oyen WJG, Corstens FHM, Storm G, In vivo applications of PEG liposomes: unexpected observations, Crit Rev Ther Durg Carrier Syst 18(6):551-66 [2001]; Oussoren C, Storm G, Liposomes to target the lymphatics by subcutaneous administration, Adv Drug Deliv Rev 50(1-2):143-56 [2001]; Bestman-Smith J, Gourde P, Desormeaux A, Tremblay MJ, Bergeron MG, Sterically stabilized liposomes bearing anti-HLA-DR antibodies for targeting the primary cellular reservoirs of HIV-1, Biochim Biophys Acta 1468(1-2):161-74 [2000]; Bestman-Smith J, Desormeaux A, Tremblay MJ, Bergeron MG, Targeting cell-free HIV and virally-infected cells with anti-HLA-DR immunoliposomes containing amphotericin B, AIDS 14(16):2457-65 [2000]; Mayer LD, Hope MJ, Cullis PR. Vesicles of variable sizes produced by a rapid extrusion procedure, Biochim Biophys Acta 858: 161-168 [1986]; Kinman, L. et al., Lipid-drug associations enhanced HIV protease inhibitor indinovir localization in lymphoid tissues and viral load reduction: a proof of concept study in HIV-infected macaques, J AIDS [2003; in press]; Harvie P, Desormeaux A, Gagne N, Tremblay M, Poulin L, Beauchamp D, Bergeron MG, Lymphoid tissues targeting of liposome-encapsulated 2',3'-dideoxyinosine, AIDS 1995 Jul;9(7):701-7 [1995]; U.S. Patent No. 5,773,027; U.S. Patent No. 5,223,263; WO 96/10399 A1).

Some useful methods of liposome preparation include extrusion, homogenization, remote loading, and reverse phase evaporation. In extrusion, a lipid film composed of phospholipids by themselves, or in combination with cholesterol, is formed by evaporating the organic solvent (such as chloroform) from the lipid solution. Hydrophobic active agents are added to the lipid solution prior to solvent evaporation. For entrapment of water soluble active agents, the dry lipid film is hydrated with and isotonic aqueuous solution containing the active agent by agitation (ultrasound, vortex, motorized stirrer, etc.). The lipid suspension is frozen and thawed three or 4 times. The suspension is then passed through a series of polycarbonate filters containing pores of a defined diameter, such as 0.8 μm, 0.4 μm, 0.2 μm, or 0.1 μm. For water soluble active agents, unencapsulated active agents are removed by gel permeation column chromatography, dialysis or diafiltration. The liposomes can be sterile-filtered (e.g., through a 0.22 μm filter).

A cryoprotectant, such as lactose, glucose, sucrose can be added to the sterile liposomes as long as isotonicity is maintained. The liposomes can then be frozen and lyophilized and stored indefinitely as a lyophilized cake. (E.g., Mayer LD, Hope MJ, Cullis PR. Vesicles of variable sizes produced by a rapid extrusion procedure, Biochim Biophys Acta 858: 161-168 [1986]; Tsvetkova NM et al. Effect of sugars on headgroup mobility in freeze-dried dipalmitoylphosphatidylcholine bilayers: solid-state 31P NMR and FTIR studies, Biophys J 75: 2947-2955 [1998]; Crowe JH, Oliver AE, Hoekstra FA, Crowe LM. Stabilization of dry membranes by mixtures of hydroxyethyl starch and glucose: the role of vitrification, Cryobiology 35: 20-30 [1997]; Sun WQ, Leopold AC, Crowe LM, Crowe JH. Stability of dry liposomes in sugar glasses, Biophys J 70: 1769-1776 [1996]).

Homogenization is suited for large scale manufacture. The lipid suspension is prepared as described above. Freeze and thaw steps on a large scale may be a problem. The diameter of the liposomes is reduced by shooting the lipid suspension as a stream either at an oncoming stream of the same lipid suspension (microfluidization) or against a steel plate (gualinizaiton). This later technology has been used by the dairy industry for homogenization of milk. Untrapped water soluble active agents are removed by diafiltration. Hydrophobic active agents are completely entrapped and there usually is no free active agent to be removed. (E.g., Paavola A, Kilpelainen I,

Yliruusi J, Rosenberg P, Controlled release injectable liposomal gel of ibuprofen for epidural analgesia, Int J Pharm 199: 85-93 [2000]; Zheng S, Zheng Y, Beissinger RL, Fresco R, Liposome-encapsulated hemoglobin processing methods, Biomater Artif Cells Immobilization Biotechnol 20: 355-364 [1992]).

Another method of active agent entrapment is remote loading. The active agent to be entrapped must carry a charge. The degree of protonation or deprotonation is controlled by the pK of the ionizable group. A conjugate acid or base is trapped inside the liposomes. The ionizable active agent is added to the outside of the liposomes. The pH is dropped such that the active agent serves as a neutralizing salt of the ionizable substance trapped inside the liposomes. The counterion to the entrapped ionizable molecule can diffuse out of the liposomes due to the change in pH. This creates a gradient with sufficient energy to cause the active agent to diffuse into the liposomes. An example is the loading of doxorubicin into preformed liposomes.

In reverse phase evaporation, a lipid film is solubilized in diethylether to a final concentration of typically about 30 mM. Typically, one part water with entrapped active agent is added to 3 parts ether lipid solution. Energy in the form of sonication is applied forcing the suspension into a homogeneous emulsion. After a stable emulsion has been formed (does not separate out after standing for 1 to 3 hours), the ether is removed by evaporation, typically yielding liposomes with about a 200 nm diameter and a high trapping efficiency.

Ethanol/Calcium liposomes for DNA Entrapment, typically yielding liposomes 50 nm in diameter, are prepared by any of the above methods (extrusion, homogenization, sonication). The liposomes are mixed with plasmid DNA plus 8 mM calcium chloride. Ethanol is typically added to the suspension to yield a concentration of about 40%. The ethanol is removed by dialysis and the resultant liposomes are generally less than 200 nm in diamter with about 75% of the DNA entrapped in the liposomes.

For cellular targeting, in accordance with the present invention, liposomes can be prepared by any of the above methods. The liposomes can contain a lipid to which proteins can be crosslinked. Examples of these lipids are: N-glutaryl-phosphatidylethnaolamine, N-succinyl-phospatidyethanolamine, Maleimido-phenyl-butyryl-phosphatidylethanolamine, succinimidyl-

acetylthioacetate-phosphatidylethanolamine, SPDP-phosphatidlyethnaolamine. The glutaryl and succinimidyl phosphosphatidylethanolamine can be linked to a nucleophile, such as an amine, using cyclocarbodiimide. The maleimido, acetylthioacetate and SPDP phosphatidylethanolamines can be reacted with thiols on the proteins, peptides or small molecular weight ligands (<1000 gm/mole). The protein can be derivatized to the liposomes after formation. Underivatized protein can be removed by gel permeation chromatography. Peptides and low molecular weight ligands can be derivatized to the lipids and added to the organic lipid solution prior to formation of the lipid film.

In accordance with the present invention, examples of useful lipids include any vesicle-forming lipid, such as, but not limited to, phospholipids, such as phosphatidylcholine (hereinafter referred to as "PC"), both naturally occurring and synthetically prepared, phosphatidic acid (hereinafter referred to as "PA"), lysophosphatidylcholine, phosphatidylserine (hereinafter referred to as "PS"), phosphatidylethanolamine (hereinafter referred to as "PE"), sphingolipids, phosphatidyglycerol (hereinafter referred to as "PG"), spingomyelin, cardiolipin, glycolipids, gangliosides, cerebrosides and the like used either singularly or intermixed such as in soybean phospholipids (e.g., Asolectin, Associated Concentrates). The PC, PG, PA and PE can be derived from purified egg yolk and its hydrogenated derivatives.

Optionally, other lipids such as steroids, cholesterol, aliphatic amines such as long-chained aliphatic amines and carboxylic acids, long chained sulfates and phosphates, diacetyl phosphate, butylated hydroxytoluene, tocopherols, retinols, and isoprenoid compounds can be intermixed with the phospholipid components to confer certain desired and known properties on the formed vesicles. In addition, synthetic phospholipids containing either altered aliphatic portions such as hydroxyl groups, branched carbon chains, cycloderivatives, aromatic derivatives, ethers, amides, polyunsaturated derivatives, halogenated derivatives or altered hydrophilic portions containing carbohydrate, glycol, phosphate, phosphonate, quarternary amine, sulfate, sulfonate, carboxy, amine, sulfhydryl, or imidazole groups and combinations of such groups can be either substituted or intermixed with the above-mentioned phospholipids and used in accordance with the invention. Some of these components are known to increase liposomal membrane fluidity, thus entailing more efficacious uptake, others are known to have a direct effect on, e.g., tumor cells by affecting their

differentiation potential. It will be appreciated from the above that the chemical composition of the lipid component prepared by the method of the invention can be varied greatly without appreciable diminution of percentage active agent capture, although the size of a vesicle can be affected by the lipid composition.

Saturated synthetic PC and PG, such as dipalmitoyl can also be used. Other amphipathic lipids that can be used, advantageously with PC, are gangliosides, globosides, fatty acids, stearylamine, long chain alcohols, and the like. PEGylated lipids, monoglycerides, diglycerides, triglycerides can also be included. Acylated and diacylated phospholipids are also useful.

By way of further example, in some embodiments, useful phospholipids include egg phosphatidylcholine ("EPC"), dilauryloylphosphatidylcholine ("DLPC"), dimyristoylphosphatidylcholine ("DOPC"), dipalmitoylphosphatidylcholine ("DPPC"), distearoylphosphatidylcholine ("DSPC"), 1-myristoyl-2-palmitoylphosphatidylcholine ("MPPC"), 1-palmitoyl-2-myristoyl phosphatidylcholine ("PMPC"), 1-palmitoyl-2-stearoyl phosphatidylcholine ("PSPC"), 1-stearoyl-2-palmitoyl phosphatidylcholine ("SPPC"), dioleoylphosphatidylycholine ("DOPC"), dilauryloylphosphatidylglycerol ("DLPG"), dimyristoylphosphatidylglycerol ("DMPG"), dipalmitoylphosphatidylglycerol ("DPPG"), distearoylphosphatidylglycerol ("DSPG"), distearoyl sphingomyelin ("DSSP"), distearoylphophatidylethanolamine (DSPE), dioleoylphosphatidylglycerol ("DOPG"), dimyristoyl phosphatidic acid ("DMPA"), dipalmitoyl phosphatidic acid ("DPPA"), dimyristoyl phosphatidylethanolamine ("DMPE"), dipalmitoyl phosphatidylethanolamine ("DPPE"), dimyristoyl phosphatidylserine ("DMPS"), dipalmitoyl phosphatidylserine ("DPPS"), brain phosphatidylserine ("BPS"), brain sphingomyelin ("BSP"), and dipalmitoyl sphingomyelin ("DPSP").

In one embodiment, phosphatidylcholine and cholesterol are employed. However, any suitable molar ratio of a non-steroidal lipid:steroidal lipid (e.g., cholesterol) mixture can optionally be employed to promote the stability of a particular lipid-active agent complex during storage and/or delivery to a mammalian subject.

Mixing the active agent and lipids can be by any useful known technique, for example, by sonication, vortexing, extrusion, microfluidization, homogenization, use of a detergent (later removed, e.g., by dialysis). The active agent and lipid are mixed at a lipid-to-active agent molar ratio of about 3:1 to about 100:1 or higher (especially useful for relatively more toxic active agents), and more preferably about 3:1 to about 10:1, and most preferably about 5:1 to about 7:1.

For some active agents, the use of an organic solvent can facilitate the production of the lipid-active agent complex, such as a liposome. The organic solvent is removed, after the mixing of the active agent and lipids, by any suitable known means of removal, such as evaporating by vacuum, or by the application of heat, for example by using a hair dryer or oven, or hot ethanol injection (e.g., Deamer, United States Patent No. 4,515,736), as long as the lipid and active agent components are stable at the temperature used. Dialysis and/or chromatography, including affinity chromatography can also be employed to remove the organic solvent. Hydrating the active agent is performed with water or any biocompatible aqueous buffer, e.g., phosphate-buffered saline, HEPES, or TRIS, that maintains a physiologically balanced osmolarity. Rehydration of liposomes can be accomplished, simultaneously with removing the organic solvent, or alternatively, can be delayed until a more convenient time for using the liposomes. (See, e.g., Papahadjopoulos et al., United States Patent No. 4,235,871). The shelf life of hydratable (i.e., "dry") liposomes is typically about 8 months to about a year, which can be increased by lyophilization.

In one embodiment, the lipid-active agent complex is a unilamellar liposome. Unilamellar liposomes provide the highest exposure of active agent to the exterior of the liposome, where it may interact with the surfaces of target cells. However, multilamellar liposomes can also be used in accordance with the present invention. The use of PEGylated liposomes is also encompassed within the present invention.

The lipid-active agent complex, such as a liposome, is preferably, but not necessarily, about 30 to about 150 nanometers in diameter, and more preferably about 50 to about 80 nanometers in diameter.

In accordance with the present invention, the lipid-active agent complexes can be preserved for later use by any known preservative method, such as lyophilization. (E.g., Crowe et al., United

States Patent No. 4,857,319). Typically, lyophilization or other useful cryopreservation techniques involve the inclusion of a cryopreservative agent, such as a disaccharide (e.g., trehalose, maltose, lactose or sucrose).

The lipid-active agent complex, e.g., a liposome, is administered to a subject by any suitable means, for example by injection. Injection can be intrarterial, intravenous, intrathecal, intraocular, subcutaneous, intramuscular, intraperitoneal, or by direct (e.g., stereotactic) injection into a particular lymphoid tissue, or into a tumor or other lesion. Subcutaneous or intramuscular injection are preferred for introducing the lipid-active agent complex into lymphatic vessels.

In accordance with the present invention, "lymphoid tissue" is a lymph node, such as an inguinal, mesenteric, ileocecal, or axillary lymph node, or the spleen, thymus, or mucosal-associated lymphoid tissue (e.g., in the lung, lamina propria of the of the intestinal wall, Peyer's patches of the small intestine, or lingual, palatine and pharyngeal tonsils, or Waldeyer's neck ring).

Injection is by any method that drains directly, or preferentially, into the lymphatic system as opposed to the blood stream. Most preferred is subcutaneous injection, typically employing a syringe needle gauge larger than the lipid-active agent complex. Intraperitoneal injection is also useful. Typically, injection of the injectate volume (generally about 1-5 cm3) is into the subject's arm, leg, or belly, but any convenient site can be chosen for subcutaneous injection. Because active agent subcutaneously administered, in accordance with some embodiments of the present invention, enters the lymphatic system prior to entering systemic blood circulation, benefits include 1) distribution throughout the lymphoid system and localization in lymph nodes, 2) the avoiding or minimizing of protein-mediated destabilization of lipid-active agent complexes, and 3) delivery of indinavir at concentrations that cannot be achieved with a soluble form of the active agent administered by any route of administration.

Typically, in treating HIV/AIDS, the frequency of injection is most preferably once per week, but more or less (e.g., monthly) frequent injections can be given as appropriate.

For purposes of the present invention, the "active agent" is one active against an infectious agent of interest.

In a preferred embodiment, the active agent is a plant lectin. Plant lectins are a class of highly interesting substances for intraendosomal HIV inhibition. Although the present invention does not depend on any particular mechanism for its therapeutic effectiveness, the ability of plant lectins to strongly bind mannose (gp120), is thought to therapeutically interfere with the virus-cell fusion process. Examples of useful plant lectins include: the mannose-specific plant lectins from *Galanthus nivalis*, *Hippeastrum hybrid*, *Narcissus pseudonarcissus*, *Epipactis helleborine*, and *Listera ovata*, and the *N*-acetylgl ucosamine-specific lectin from *Urtica dioica* which inhibit HIV-1 and HIV-2 infection at an IC_{50} of about 0.04 to about 0.08 μg/mL (this is the concentration needed upon non-targeted delivery). An irreversible agglutination network is formed among intraendosomally stored HIV or other pathogen in reservoir cells, inactivating its pathogenic capacity. Plant lectins not only interfere strongly with both HSV-1 and HSV-2, but also CMV, RSV and influenza virus. In addition, HCV and VSV are inactivated by plant lectins derived from *Canavalia ensiformis* (similar to that shown in **Fig. 3**) and *Arceuthobium spp.* (mistletoe) (but not by animal C-type lectins such as that shown in **Fig. 2**). This may carry the same concept, even without major modification, directly to its application in other viral diseases.

Spatially, tetramers of plant lectins are typically ~0.5 Å (= 5 nm) in diameter (depending on the pH, they exist either as dimers or tetramers). Thus, many such molecules should be entrapped in, and delivered from, a 150-nm liposome to successively agglutinate endosomally stored viral reservoir.

In other embodiments of the invention, the active agent comprised by the mono- or poly-fucose targeted liposome is a drug, which can be an anti-viral drug or virostatic agent, such as, interferon, a nucleoside analog, or a non-nucleoside anti-viral drug. Examples include anti-HIV drugs (e.g.; a HIV reverse protease inhibitor), such as indinavir (aka Crixivan®, Merck & Co., Inc., Rahway, NJ; saquinavir (N-tert-butyl-decahydro-2-[2(R)- hydroxy-4-phenyl-3(S)-[[N-(2-quinolylcarbonyl)-L-asparaginyl]-amino]butyl]-(4aS,8aS)-isoquinoline-3(S)-carboxamide; MW = 670.86; aka Fortovase®, Roche Laboratories, Inc., Nutley, NJ); or nelfinavir (i.e., nelfinavir mesylate, aka Viracept®; [3S-[2(2S*, 3S*), 3a,4ab,8ab]]-N-(1,1-dimethylethyl)decahydro-2-[2-hydroxy-3-[(3-hydroxy-2-methylbenzoyl)amino]-4-(phenylthio)butyl]-3-isoquinolinecarboxamide

mono-methanesulfonate (salt), MW = 663.90 [567.79 as the free base]; Agouron Pharmaceuticals, Inc., La Jolla, CA). Nelfinavir mesylate is a white to off-white amorphous powder, slightly soluble in water at pH <4 and freely soluble in methanol, ethanol, isopropanol and propylene glycol. Other examples of antiviral drug include reverse transcriptase inhibitors, such as tenofovir disoproxil fumarate (9-[(R)-2-[[bis[[(isopropoxycarbonyl) oxy]methoxy]phosphinyl]methoxy]propyl]adenine fumarate (1:1); MW = 635.52; aka Viread®, Gilead Sciences, Foster City, CA). The anti-HIV drug can also be HIV-specific siRNA, anti-sense or sense DNA molecules.

In other embodiments, the active agent is an anticancer drug, an antifungal drug, or an antibacterial drug. In other embodiments, the active agent is an immunomodulatory agent (i.e., an immunoactivator, an immunogen, an immunosuppressant, or an anti-inflammatory agent), such as cyclosporin, steroids and steroid derivatives. Other examples of useful drugs, in accordance with the invention, include therapeutic cytotoxic agents (e.g., cisplatin, carboplatin, methotrexate, 5-fluorouracil, and amphotericin), naked DNA expression vectors, therapeutic proteins, therapeutic oligonucleotides or nucleotide analogs, interferons, cytokines, or cytokine agonists or antagonists. Also useful as a drug is a cytotoxic alkylating agent, such as, but not limited to, busulfan (1,4-butanediol dimethanesulphonate; Myleran, Glaxo Wellcome), chlorambucil, cyclophosphamide, melphalan, or ethyl ethanesulfonic acid. Such drugs or agents are particularly useful in treating conditions involving pathological proliferation of immune cells, for example, lymphoid cancers or autoimmune diseases.

Combinations of two or more different active agents, whether plant lectins and/or drugs, are also encompassed within the invention. For example, useful liposomes can comprise a combination of an anti-HIV drug and an antifungal and/or antibacterial drug, with or without a plant lectin.

1. **(Fig. 1)**.

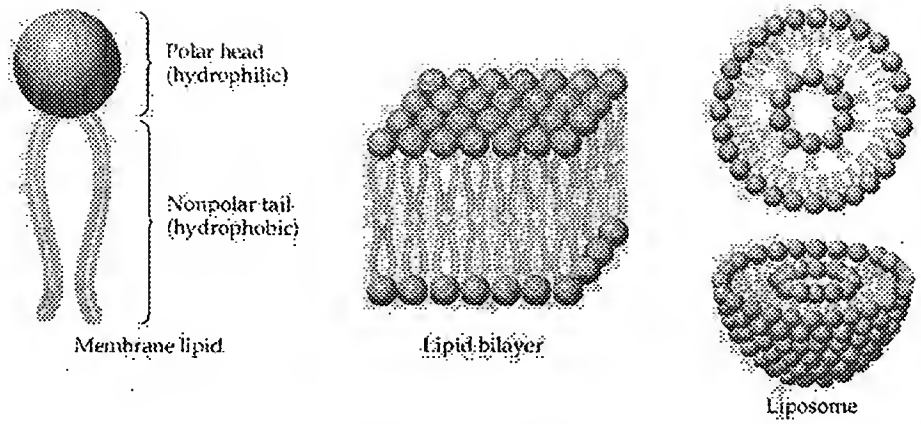

Fig. 1: Formation of liposomes. (Poly-)fucose can be linked to the polar heads of the components of the outer membrane, thus allowing targeting to C-type lectin receptors on HIV reservoir populations, including DCs, macrophages, as well as HIV-transferring mucosal and placental cells.
Source: McMurry & Castellion: Fundamentals of General, Organic and Biologic Chemistry, 3rd ed., http://cwx.prenhall.com/bookbind/pubbooks/mcmurrygob/medialib/media_portfolio/24.html

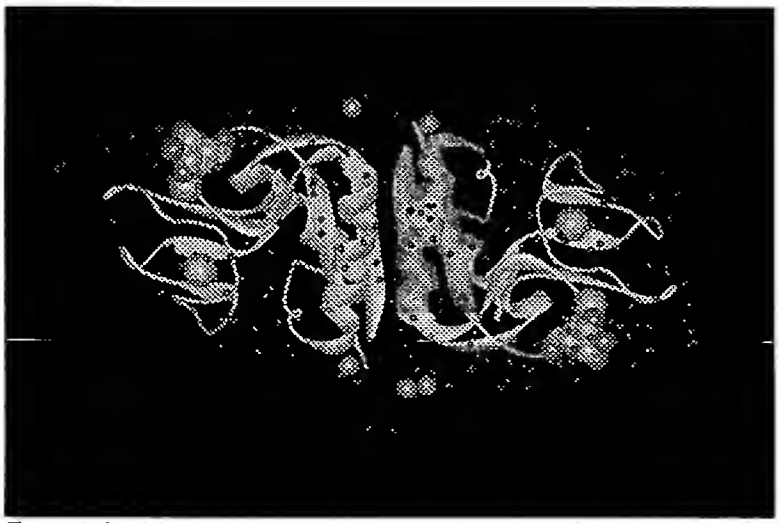

Fig. 2: Animal C-type lectin. Homodimer (of the tunicate *Polyandrocarpa misakiensis*) with bound sugar molecules.
Source: CNRS, France; http://www.cermav.cnrs.fr/cgi-bin/lectines/menu2.cgi?1TLG

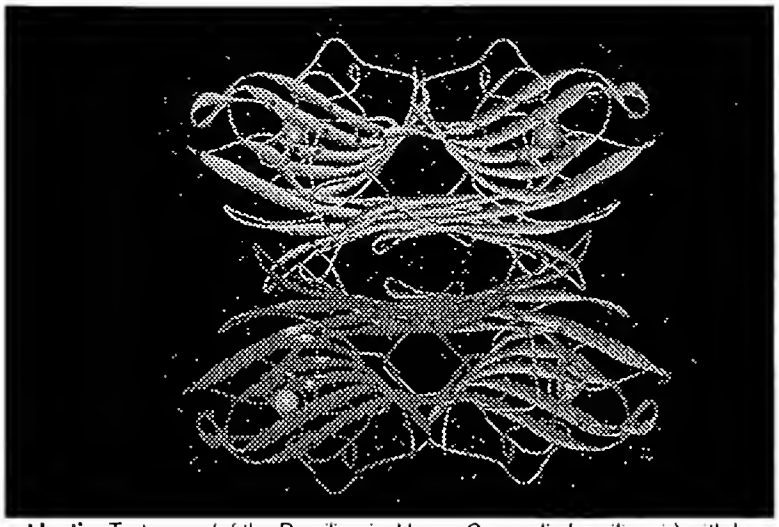

Fig. 3: Plant lectin. Tertramer (of the Brazilian jackbean *Canavalia brasiliensis*) with bound sugar molecules. Source: CNRS, France; http://www.cermav.cnrs.fr/cgi-bin/lectines/menu2.cgi?1J4S